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REGULATORY PROTEIN FROM HUMAN KERATINOCYTES

Description

[0001] The invention relates to an isolated polypeptide that is identical or similar (i.e., the same in function and effect) to a protein that occurs naturally in human keratinocytes and is increasingly expressed when the keratinocytes are in an activated state. It also relates to an isolated nucleic acid, which codes a polypeptide or protein typical for human keratinocytes, and to the use of this polypeptide and this nucleic acid for detection, in particular diagnostic, and/or therapeutic purposes, and the use of reagents, in particular recombinant vector molecules and antibodies against such molecules.

[0002] Based on prior art as currently exists, essentially pharmaceuticals with a broad range of action are used in skin treatment to influence epidermal disturbances, e.g., autoimmune dermatoses „Pemphigus vulgaris“ and „Bullous Pemphigoid“, including locally or systemically applied glucocorticoids, vitamin A acid derivatives, antimetabolites and cytostatics, or more or less non-specific measures are used in treatment, such as „dye therapy“ or „light therapy“. However, the disadvantage to all known agents or measures is that they are not very specific, and hence of course bring about numerous side effects.

[0003] The preparation of more specific agents has thus far been unsuccessful due to a basic problem that has persisted in dermatology for a long time, namely that the number of cellular target molecules (target structures, targets) which might serve as a point of attack for exerting a (specific) influence on cellular metabolism, in particular from a medical or even cosmetic standpoint, is narrowly restricted in epidermal keratinocytes.

[0004] Therefore, the object of this invention is to provide new target structures in epidermal keratinocytes that can serve as a point of attack for diagnostic, therapeutic and cosmetic agents, or generally for influencing cellular metabolism.

[0005] One solution to this object involves preparing a protein of the kind mentioned at the outset, which is upwardly adjusted given activated keratinocytes, i.e., increasingly expressed or produced, and kept at a higher concentration level, and which has the amino acid sequence indicated in either the SEQ ID NO:2 sequence protocol or

the SEQ ID NO:3 sequence protocol, or an allele or derivative obtained through amino acid substitution, deletion, insertion or inversion from one of these two amino acid sequences. In the following, the polypeptide with the SEQ ID NO:2 or SEQ ID NO:3 amino acid sequence shall also be referred to as protein pKe#122.

[0006] Another solution to this object involves preparing an isolated nucleic acid that codes a protein, which is identical or similar to a protein that occurs naturally in human keratinocytes and is increasingly expressed when the keratinocytes are in an activated state, and which has the nucleotide sequence indicated in either the SEQ ID NO:1 sequence protocol or the SEQ ID NO:4 sequence protocol, or a nucleotide sequence complementary to one of these two, or a partial sequence of one of these two indicated or complementary nucleotide sequences, or a nucleotide sequence that hybridizes wholly or in part with one of these aforementioned nucleotide sequences, wherein „U“ can take the place of „T“ in these two sequence protocols. This group of nucleic acids or nucleotide sequences according to the invention also includes in particular splice variants and sense or antisense oligonucleotides, which hybridize with the nucleotide sequence indicated in the SEQ ID NO:1 sequence protocol or the SEQ ID NO:4 sequence protocol, preferably identical or complementary to at least one of these two.

[0007] As a result, the invention also encompasses proteins or polypeptides of the kind mentioned at the outset, which have an amino acid sequence that results from such a splice variant, in particular the splice variant of an mRNA, which is identical or complementary to the nucleotide sequence indicated in the SEQ ID NO:1 sequence protocol or the SEQ ID NO:4 sequence protocol.

[0008] The sense or antisense oligonucleotides according to the invention encompass at least 6, preferably 8 to 25 nucleotides.

[0009] The term „hybridized“ relates to the hybridization procedures known in the art under conventional, in particular also under highly stringent hybridization conditions. The expert selects the specific hybridization parameters based on the used nucleotide sequence and his or her general technical knowledge (compare: *Current Protocols in Molecular Biology*, Vol. 1, 1997, John Wiley & Sons Inc., Suppl. 37, Chapter 4.9.14). The nucleic acid(s) according to the invention can be obtained from both a natural source or synthetically or semi-synthetically. Its presentation as cDNA has proven to be particularly effective in practice.

[0010] The polypeptide that has the amino acid sequence according to SEQ ID NO:2 or SEQ ID NO:3 and is coded by the nucleic acid indicated in the SEQ ID NO:1 sequence protocol or the SEQ ID NO:4 sequence protocol, and that is referred to as protein pKe#122 below, is upwardly adjusted in human epidermal keratinocytes, namely increasingly expressed (produced) and kept at a significantly higher concentration level in comparison to the initial state if these cells are in the „activated“ state, i.e., in a state of proliferation and/or migration, among others, e.g., after an accidental skin injury or given an autoimmunologically induced bullous dermatoses „Pemphigus vulgaris“ (triggered by autoantibodies against desmosomes) and „Bullous Pemphigoid“ (triggered by autoantibodies against hemidesmosomes). The activated state of the human epidermal keratinocytes is also manifested in an elevated expression of known activation markers uPA (urokinase-type plasminogen activator) and uPA-R (receptor for urokinase-type plasminogen activator) relative to the resting state (initial state), and can be qualitatively and quantitatively detected based on these markers. (compare: Schäfer B.M., Reinartz J., Bechtel M.J., Inndorf S., Lang E., und Kramer M. D., 1996: *Dispase mediated basal detachment of cultured keratinocytes induces urokinase-type plasminogen activator (uPA) and its receptor (uPA-R, CD87)*, *Exp. Cell Res.* **228**, pp. 246-253).

[0011] Protein pKe#122 has a serine/threonine-kinase motif, several (four) tyrosine kinase phosphorylation motifs and a kinase domain with an ATP binding site. It is obviously involved in signal transduction processes, very probably has a serine/threonine-kinase function, and plays a presumed role in the formation of cell-cell and/or cell-matrix connections, and/or of desmosomes and/or hemidesmosomes.

[0012] It is known in prior art that serine/threonine-kinases influence the function of cell-cell and cell-matrix contacts in keratinocytes. S. Blum and coauthors demonstrated that the localization of specific cell contact molecules of the *Zonula adherens* can be influenced by activating or inactivating the „protein kinase C (PKC)“-type serine/threonine-kinase (compare Blum S., Ness W., Petrow W., Achenbach F., 1994: *Localization of protein kinase C in primary cultures of human keratinocytes in relation to cell contact proteins.* *Cell Sig.* **6**:157-165). In addition, M. Serres and coauthors showed that treating keratinocytes in a cell culture (HaCaT cells) with the serine/threonine-phosphatase inhibitors okadaic acid, calyculin and Pefabloc<sup>TM</sup> results in a loss of cell-cell connections on the one hand, and in an increased serine/threonine phosphorylation of the linker protein  $\beta$ -catenine involved in cellular adhesion (compare

Serres M., Grangeasse C., Haftek M., Durocher Y., Duclos B., Schmitt D., 1997: *Hyperphosphorylation of  $\beta$ -Catenin on serine-threonine residues and loss of cell-cell contacts induced by calyculin A and okadaic acid in human epidermal cells. Exp. Cell. Res. 231: 163-172*). These in-vitro findings were confirmed on epidermal keratinocytes of explanted human skin: a clear disruption of epidermal cell/cell connections with acantholysis occurred after the application of okadaic acid (2  $\mu$ M, 24 hours). Neither PKC activators (e.g., bryostatin-1 or TPA=PMA=phorbol myristate acetate) and PKC inhibitors (sphingosine, staurosporine, chelerythrine, H7=1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) nor inhibitors or activators of protein kinase A (PKA), nor tyrosine-kinase and phosphatase inhibitors or less specific phosphatase inhibitors had such an effect on the cells.

[0013] Serine/threonine kinases also play an important role in the formation of hemidesmosomes (compare Mainiero F., Pepe A., Wary K.K., Spinardi L., Mohammadi M., Schlessinger J. Giancotti F.G., 1995: *Signal transduction by the  $\alpha 6 \beta 4$  integrin: distinct  $\beta 4$  subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes*. EMBO J. 14:4470-4481).

[0014] The isolated preparation of protein pKe#122, namely the description of nucleotide sequences that code this protein, and the indication of (one of) its amino acid sequence(s) make it possible to exert a targeted influence on the metabolism of physiologically active or activated keratinocytes, and of course of other cells that express protein pKe#122, in particular for purposes of medical and cosmetic therapy.

[0015] The invention also relates to recombinant DNA vector molecules, which encompass a nucleic acid according to the invention, and which have the ability to express a protein that occurs in human keratinocytes and is increasingly expressed when the keratinocytes are in an activated state, in particular protein pKe#122, in a prokaryotic or eukaryotic cell. The DNA vector molecules preferably involve the plasmid pUEX-1 and/or the plasmid pGEX-2T and/or the plasmid pBK-CMV and/or the plasmid pHR 2 (a derivative of Bluescript KS [Stratagene, Heidelberg], contains the human keratin-14 promotor), since these vectors have proven to be highly suitable in practice. While the eukaryotic cell includes in particular cells from cell cultures, e.g., COS cells, the respective cell can also be a constituent of a living organism, e.g., a transgenic mouse.

[0016] Therefore, the invention also encompasses transformed host cells that contain a nucleic acid according to the invention that is linked with an activatable promotor, which is contained in these cells naturally or as the result of recombination, and that (consequently) have the ability to express a protein that occurs naturally in human keratinocytes and is increasingly expressed when the keratinocytes are in an activated state, in particular protein pKe#122.

[0017] The invention also relates to the use of a nucleic acid according to the invention or a vector molecule according to the invention to manufacture transgenic mammals, in particular mice or rats.

[0018] The transfectants according to the invention open up an opportunity for research and development work aimed at further clarifying the protein pKe#122-induced changes in cell morphology and cellular base functions such as proliferation, adhesion, migration and differentiation, in particular with an eye toward answering the question as to whether protein pKe#122 itself possesses a „pathogenic“ activity.

[0019] The object of this invention also relates to a reagent for the indirect detection of a protein that is encountered in human keratinocytes and increasingly expressed when the keratinocytes are in an activated state, in particular protein pKe#122, wherein this reagent is characterized by the fact that it encompasses at least one nucleic acid according to the invention. In this context, „for the indirect detection“ implies that the protein-coding mRNA is actually directly detected, and hence the protein is only indirectly detected (by means of this mRNA).

[0020] Protein pKe#122 and the polypeptides related thereto, i.e., to the amino acid sequence indicated in the SEQ ID NO:2 sequence protocol or SEQ ID NO:3 sequence protocol, specifically the polypeptides that can be derived through substitution, deletion, insertion and/or inversion from one of these amino acid sequences according to SEQ ID NO:2 or SEQ ID NO:3, or that have an amino acid sequence resulting from a splice variant of an mRNA, which is identical or complementary to the nucleotide sequence indicated in the SEQ ID NO:1 sequence protocol or the SEQ ID NO:4 sequence protocol, or to a partial sequence of these nucleotide sequences, or at least hybridized, offer numerous applications in the area of dermatological research and development. In particular, antibodies can be developed against these polypeptides or proteins, which then can be correspondingly modified for use either as diagnostic or therapeutic agents, or as cosmetic agents („cosmeceuticals“).

[0021] Consequently, the invention also encompasses the use of such a protein or polypeptide for manufacturing a (monoclonal, polyclonal or recombinant) antibody against this polypeptide, the aforementioned antibody itself, and also its use for the diagnostic and/or therapeutic treatment of dermatological diseases, for the cosmetic treatment of the epidermis, and for the diagnostic, therapeutic and/or cosmetic treatment of other tissues or organs that express protein pKe#122.

[0022] According to more recent scientific knowledge, sense and/or antisense oligonucleotides are also possible as active agents for pharmacotherapy (compare G. Hartmann et al. 1998: *Antisense Oligonucleotides*, Deutsches Ärzteblatt 95, Issue 24, C1115-C1119), and also as active agents with a fundamentally new operating principle in pharmacotherapy.

[0023] Therefore, the present invention also relates to the use of sense or antisense oligonucleotides according to the invention for diagnostic and/or therapeutic treatment, in particular of dermatological diseases, or for the cosmetic treatment in particular of the epidermis.

[0024] One technically and economically important potential application for a polypeptide according to the invention or a nucleic acid according to the invention also involves not least the fact that such a molecule can be used in a screening procedure to isolate materials from a very high number of provided materials that specifically bind to the respective nucleic acid or respective polypeptide. These substances can then serve as the parent material (lead structure) for the development of substances for use in pharmacology, and hence offer the preconditions for the development of alternative pharmaceuticals for diagnosis and therapy, in particular with respect to the dermatological diseases mentioned at the outset.

[0025] In this regard, the invention also relates to the application of a polypeptide according to the invention or a nucleic acid according to the invention for identifying substances that can be used in pharmacology, which bind to the polypeptide or nucleic acid, thereby influencing its/their function and/or expression, in particular exerting an inhibiting or activating effect.

[0026] The invention will be explained in greater detail below based on manufacturing and application examples.

### Detailed Description

Example 1: Manufacture of Protein pKe#122

A) Extraction or Manufacture of a Polynucleotide that Codes Protein pKe#122

[0027] The polynucleotide source consisted of human epidermal keratinocytes of a cell culture or cell culture model described extensively in the publication of Schäfer B.M., Reinartz J., Bechtel M.J., Inndorf S., Lang E., und Kramer M. D., 1996: *Dispace mediated basal detachment of cultured keratinocytes induces urokinase-type plasminogen activator (uPA) and its receptor (uPA-R, CD87)*, *Exp. Cell Res.* **228**, pp. 246-253. Reference is hereby made expressly to the content of this publication. This cell culture or cell culture model is characterized by the fact that it makes it possible to convert keratinocytes from the resting  $[uPA^-/uPA-R^-]$  to the activated  $[uPA^+/uPA-R^+]$  state through enzymatic disruption of the cell/matrix contacts, i.e., dispace-induced detachment of the keratinocytes from the culture matrix. The induction of the activated state is reversible: the (renewed) formation of a confluent (=grown to maximal density), multilayered cell aggregate consisting of differentiated keratinocytes results in the downward adjustment of uPA and uPA-R, i.e., the slowing of production and setting to a lower concentration level (see the publication of Schäfer B.M., Stark H.J., Fusenig N.E., Rodd R.F., Kramer M.D., 1996 *Differential expression of urokinase-type plasminogen activator (uPA), its receptor (uPA-R), and inhibitor type-2 (PAI-2) during differentiation of keratinocytes in an organotypic coculture system*, *Exp. Cell Res.* **220**:415-423).

[0028] Cells in this cell culture or cell culture model shall also be referred to as NHEK below (=„normal human epidermal keratinocytes“).

[0029] The following measures were implemented for preparing the cell culture or cell culture model: Human epidermal keratinocytes obtained from a skin biopsy were trypsinated overnight at 4°C and then cultivated in Petri dishes or 175 cm<sup>2</sup> culture flasks according to the „feeder-layer“ technique of J.G. Rheinwald and H. Green (1975, *Cell* **6**, 331-334) for a duration of 8 days in Dulbecco's modified Eagle's Medium (DMEM) with a content of 10% (vol./vol.) fetal calf serum (FCS) and added adenine hemisulfate, insulin, transferrin, triiodothyronine, hydrocortisone, Forskolin, epidermal growth factor (EGF) and antibiotics (penicillin, streptomycin and gentamycin) under differentiation conditions, namely elevated calcium levels (37 °C, 7% CO<sub>2</sub>). Therefore, cultivation took place under conventional conditions common in prior art. Under these conditions,

keratinocytes form confluent two to three-layer „epidermis equivalents“, or keratinocyte „sheets“.

[0030] These epidermis equivalents or keratinocyte sheets were detached from the culture matrix in a 30-minute treatment with dispase II (2.4 mg/ml in DMEM without FCS), washed twice in DMEM and then incubated in complete, conditioned DMEM for a duration of 4 or 8 hours. Incubation in conditioned DMEM took place to preclude the influence of fresh FCS. During incubation, the expression of known activation markers uPA and uPA-R was upwardly adjusted in these floating keratinocyte sheets, as was protein pKe#122 described for the first time herein. The uPA/uPA-R upward adjustment could be detected by means of known techniques, such as enzyme linked immunosorbent assay (ELISA), in-situ hybridization and immune fluorescence. The entire RNA was extracted from the incubated cells ("RNA-Clean" kit, AGS company in Heidelberg) using the guanidinium-thiocyanate-phenol-chloroform extraction method known in the art (compare Chromczynski P. and Sacchi N., 1986: *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction*. Anal. Biochem. 162:156-159). The mRNA was isolated from the entire RNA through binding on poly-T coated beads. This mRNA was used as the starting material for the ensuing step of subtraction cloning.

[0031] mRNA was isolated from adherent keratinocyte sheets for use in control tests or for comparison preparations, specifically according to the same procedural pattern described above, except that a dispase inhibitor, e.g., phosporamidone (100 µg/ml), was additionally applied to the dispase for the duration of dispase treatment.

[0032] The principle of subtraction cloning was used to establish a gene bank, which preferably contained cDNA of the dyshesion-induced gene, i.e., of those genes that were increasingly expressed after detachment of the keratinocyte sheets in the latter (or their cells). To this end, the mRNA obtained from the cells of the adherent keratinocyte sheets was again bound to poly-T coated beads, rewritten into single-strand cDNA on the latter, and then hybridized against the mRNA of detached, i.e., non-adherent keratinocyte sheets. Those mRNA molecules that were expressed only in the non-adherent state, i.e., after dyshesion, and hence found no hybridization partner, remained behind as a supernatant. They were rewritten into cDNA and cloned in cloning vector pUEX-1.



[0033] For purposes of verification, the resultant gene bank was then also subjected to a southernblot procedure with [ $^{32}\text{P}$ ]-marked cDNA of adherent and non-adherent keratinocyte sheets. Those cDNA, or rather the host cell clones containing them, here the *E. coli* strain MC1061, which exhibited a distinct upward adjustment after dyshesion, were subsequently cultivated or multiplied overnight at 30°C under conventional culture conditions. The plasmid DNA (pUEX1-cDNA) were prepared from these *E. coli* clones. The cDNA fragments were cut out of the pUEX1 vector and were [ $^{32}\text{P}$ ]-marked by means of random priming. The marked cDNA was used as a probe in northernblots with RNA from adherent and non-adherent keratinocyte sheets. The clones containing cDNA, which revealed no or only a slight signal with the RNA of adherent keratinocytes when used as a probe in the northernblot procedure, but exhibited a distinct signal with RNA of non-adherent keratinocytes, were selected for the ensuing step of sequencing.

[0034] While sequencing the respective clones by means of „non-radioactive cycle sequencing“, which is a modification of the sequencing method according to Sanger and has in the meantime become a common method in prior art, the gene with the nucleotide sequence according to SEQ ID NO:1 and SEQ ID NO:4 was found. This gene and the accompanying protein were designated pKe#122. More detailed analyses of the mRNA that belongs to gene pKe#122, i.e., is pKe#122-specific (from dissolved, i.e., non-adherent keratinocyte sheets), provided information as to the fact that this mRNA has a size of roughly 4.8 kb, and exhibits an upward regulation after disperse-induced detachment. Fig. 1 shows the results of a Northern blot, which was performed with mRNA from keratinocyte sheets (a) immediately after or (b) four hours after disperse-induced detachment and with [ $^{32}\text{P}$ ]-marked pKe#122 cDNA. This result indicates that little pKe#122 mRNA was present, or at least detectable, immediately after detachment, while large quantities were present four hours later (a broad, color-intensive band), namely in the molecular weight zone of about 4.8 kb.

[0035] The nucleotide sequence of the pKe#122 gene has a stop codon at the 3' end at position 2373-2375 according to SEQ ID NO:1, and hence at position 2472-2474 according to SEQ ID NO:4, which stipulates the probable location of the transcription end, and which is followed by a sequence very similar to the „polyadenylation site“ (AATAAA), namely AATAA, exactly 28 nucleic acids before the poly-A site.

[0036] As a result with respect to the overall structure of the pKe#122 gene, or in general of a polynucleotide that codes for protein pKe#122, we find that this gene or

polynucleotide has the nucleotide sequence indicated in the SEQ IN NO:1 sequence protocol or the SEQ IN NO:4 sequence protocol, or a partial sequence of one of these two nucleotide sequences, or encompasses a nucleotide sequence or consists of one that is complementary to one of these indicated nucleotide sequences or one of their partial sequences, or that this gene or polynucleotide is wholly or partially hybridized with the nucleotide sequence indicated in the SEQ ID NO:1 sequence protocol or the SEQ ID NO:4 sequence protocol or with a partial sequence of one of these two nucleotide sequences, or with a sequence complementary to these indicated nucleotide sequences or their partial sequences, wherein „U“ can take the place of „T“ in the SEQ ID NO:1 and SEQ ID NO:4 sequence protocols, and that an mRNA corresponding or homologous to a cDNA of approx. 4.8 kb is read from this gene or polynucleotide.

B) Derivation of the Amino Acid Sequence and Characterization of the pKe#122 Protein based on the polynucleotide coded for this purpose (pKe#122 gene)

[0037] Based on the genetic code and using a computer-assisted procedure (program: *HUSAR = Heidelberg Unix Sequence Analysis Resources*, Version 4.0, German Cancer Research Center, Heidelberg, 1997), an amino acid sequence indicated in the SEQ ID NO:2 and/or SEQ ID NO:3 sequence protocol was derived from the nucleotide sequence according to the SEQ ID NO:1 and SEQ ID NO:4 sequence protocol. A structural analysis of these amino acid sequences according to the SEQ ID NO:2 and SEQ ID NO:3 sequence protocol with this very program yielded the following information, which is valid for both amino acid sequences:

- the amino acid sequence from position 40 to 63 (LGKGNFAVVKLARHRVTK TQVAIK) according to SEQ ID NO:2, and consequently from position 73 to 96 according to SEQ ID NO:3 corresponds to known protein kinase motifs with ATP binding site,
- the amino acid sequence from position 152 to 164 (IVHRDLKTENLLL) according to SEQ ID NO:2, and consequently from position 185 to 197 according to SEQ ID NO:3 corresponds to known serine/threonine protein kinase motifs,
- the amino acid sequences from position 238 to 240 (TLR), from position 475 to 477 (TGR), from position 485 to 487 (STR) and from position 600 to 603 (TTR) according to SEQ ID NO:2 and from position 271 to 273 (TLR), from position 508 to 510 (TGR), from position 518 to 520 (STR) and from position 633 to 635 (TTR) according to SEQ ID NO:3 correspond to known phosphorylation sites for protein kinase C,

- the amino acid sequence from position 138 to 156 (WQILSAVEYCHDHHIVHRD) according to SEQ ID NO:2 and consequently from position 171 to 189 according to SEQ ID NO:3 represents the pKe#122-1 peptide, against which the anti-peptide antibody „anti-pKe#122-1“ was produced in a rabbit (compare Example 2),
- the amino acid sequence from position 481 to 499 (LAEVSTRLSPLTAPCIVVS) according to SEQ ID NO:2 and consequently from position 514 to 532 according to SEQ ID NO:3 represents the pKe#122-2 peptide, against which the anti-peptide antibody „anti-pKe#122-2“ was produced in a rabbit (compare Example 2).
- the amino acid sequence from position 339 to 352 (NHFAAIYYLLLERL) according to SEQ ID NO:2 and consequently from position 372 to 385 according to SEQ ID NO:3 represents the pKe#122-3 peptide, against which the anti-peptide antibody „anti-pKe#122-3“ was produced in a rabbit (compare Example 2).
- the amino acid sequence from position 614 to 625 (GLARQVCQVPAS) according to SEQ ID NO:2 and consequently from position 647 to 658 according to SEQ ID NO:3 represents the pKe#122-4 peptide, against which the anti-peptide antibody „anti-pKe#122-4“ was produced in a rabbit (compare Example 2).

[0038] In Fig. 2 (for SEQ ID NO:2 ) and Fig. 14 (for SEQ ID NO:3), these structural data for protein pKe#122 are shown diagrammatically. Fig. 2A and Fig. 14A show the protein kinase motif with ATP binding sites, the serine/threonine protein kinase motif and the four phosphorylation sites for protein kinase C, while Fig. 2B and Fig. 14B show the sequence segments against which anti-peptide antibodies were produced in rabbits.

[0039] Example 2: Use of the Amino Acid Sequence of the pKe#122 Protein for the Manufacture of Polyclonal Anti-Peptide Antibodies

[0040] A computer-assisted antigenicity analysis was performed using the computer program mentioned in Example 1 to select areas from the amino acid sequence according to SEQ ID NO:2 that appeared suitable for the production of polyclonal anti-peptide antibodies. These areas were synthesized according to the known

„multiple-antigenic-peptide“ procedure (compare: Posnett D.N., Tam J.P., 1989: *Multiple antigenic peptide method for producing antipeptide site-specific antibodies*. Methods-Enzymol. 1998; 178: 739-746) in the form of separate peptides (pKe#122-1 to -4, compare Fig. 2) with a molecular weight of approx. 10-15 kD. These peptides were used without the addition of a carrier substance for the adjuvant-assisted immunization of rabbits. The details of this peptide manufacturing procedure and this immunization procedure are generally known in prior art. The pre- and post-immunoserum were tested for reactivity with the respective peptides and comparison peptides used for the immunization by means of the generally known enzyme-linked immunosorbent assay (ELISA). A clear immunization against peptides pKe#122-1, -122-2 and -122-4 could be detected. To purify the polyclonal antibodies, the post-immunoserum was initially subjected to ammonium sulfate precipitation, which enriched the IgG fraction. Immune-affinity chromatography was then carried out with this enriched IgG fraction. To this end, the four peptides pKe#122-1 to -4 used for immunization were immobilized on sepharose 4B, and this peptide-sepharose 4B conjugate was used in immune-affinity chromatography. This resulted in three largely pure anti-peptide IgG fractions, namely anti-peptide pKe#122-1, anti-peptide pKe#122-2 and anti-peptide pKe#122-4. These three affinity-purified antibody fractions showed a clear immune reaction with the respectively corresponding antigen peptide. Table 1 shows these results.

[0041] The immune serum against peptide pKe#122-1, the polyclonal antibody anti-pKe#122-1, was also used to test cell lysates of the keratinocyte line HaCaT and keratinocyte sheets 8 hours after detachment with dispase (=non-adherent keratinocyte sheets) in the Western-blot procedure for the expression of the pKe#122 protein. A band with a molecular weight of approx. 70-85 kD was detected in both the HaCaT cells and the cells of the detached (non-adherent) keratinocyte sheets. The Western-blot used in the experiment is described in detail in Example 3B(2) and depicted on Fig. 3. It exhibits a protein measuring approx. 70-85 kD in both HaCaT cells and in the non-adherent keratinocyte sheets.

[0042] The polyclonal anti-peptide antibody pKe#122-1 was additionally tested in the immunoblot procedure with the recombinant approx. 100 kD GST-pKe#122 fusion protein (fraction 85 in Fig. 10B) described here in Example 5. The polyclonal anti-peptide antibody pKe#122-1 reacted with the fusion protein. This positive reaction was confirmed by a comparison with control tests, in which an anti-GST antibody or normal rabbit IgG was used instead of the anti-peptide antibody pKe#122-1. The results

are presented in Table 1 and on Fig. 4. Trace „a“ on Fig. 4 shows the control batch with normal goat IgG, while trace „d“ on Fig. 4 shows the batch with rabbit anti-pKe#122-1.

[0043] Example 3: Use of the pKe#122 protein either against the protein or against the mRNA of the pKe#122-oriented reagents for detecting the activated state of human epidermal keratinocytes

#### A) Used Keratinocytes

[0044] The test cells or target cells (=target object cells) were HaCaT cells and human epidermal keratinocytes of the cell culture or the cell culture model (NHEK) that was extensively described in the publication by B.M. Schäfer and coauthors (loco citato) and is briefly summarized here in Example 1 (A). Express reference will be made to the content of this publication at this juncture too. In addition, skin biopsies were analyzed for the expression of the pKe#122 protein.

#### B) Detection procedures based on the use of antibodies oriented against the pKe#122 protein.

##### 1. Immunohistology

[0045] A cryotom was used to manufacture 5 µm thick frozen sections of tissues from skin biopsies of clinically unpathological, normal skin and clinically pathological, lesional skin owing to the diseases Pemphigus vulgaris, Bullous Pemphigoid and Psoriasis vulgaris. These are dried at room temperature and fixed in 100% acetone (100% methanol, 100% ethanol or 4% paraformaldehyde can be used instead of acetone). The sections are then treated according to the „blocking procedure“ known in prior art to block non-specific binding sites for the antibody. In this example, two blocking steps are performed: (1) blocking with avidin/biotin and (2) blocking with normal serum. In the first blocking step, the avidin/biotin blocking was performed using the avidin-biotin blocking kit from Vector Laboratories according to the manufacturer's instructions, i.e., incubation was performed at room temperature initially for 15 minutes with the avidin finished solution, and then 15 minutes with the biotin finished solution. Subsequently, the sections were incubated with 10 vol.% normal serum in PBS (normal serum of species from which the second antibody originates, here goat normal serum; PBS = phosphate buffered saline, pH 7.2-7.4) for 15 minutes at room temperature.

[0046] After blocking, the sections in PBS are incubated for 1 hour at room temperature with a content of 5 µg/ml anti-peptide pKe#122-1. To remove the unbound antibody, the sections are then washed in PBS with a content of 0.2% (weight/volume) bovine serum albumin. This is followed by incubation, for example with a biotin-labelled antibody from the goat against rabbit IgG (1:500 diluted in PBS/0.2% BSA; 30 minutes at room temperature), another washing step and the application of a streptavidin labelled with the fluorescent dye Cy3 (1:1,000 in PBS/0.2% BSA diluted). A fluorescent dye other than Cy3 can also be used to mark the streptavidin, e.g., FITC. After the last washing step, the sections are covered with a covering agent, e.g., elvanol or histogel, and then analyzed and evaluated under a fluorescence microscope. Fig. 5 shows the results obtained from an immune fluorescence detection performed in this manner: The anti-pKe#122-1 IgG antibody stains keratinocytes on normal skin sections in the area of the epidermal basal membrane zone (Fig. 5A). When dying biopsies of lesional skin caused by the diseases Pemphigus vulgaris (Fig. 5B), Bullous Pemphigoid (Fig. 5C) or Psoriasis vulgaris (Fig. 5D), a distinctively strong coloration is observed in epidermal keratinocytes, in particular in the area of epidermal lesions. Hence, an increased expression and evident upward adjustment of the pKe#122 protein took place there.

## 2. Immunoblot („Western-blot“) and Dot blot

[0047] Fig. 3 shows the detection of the pKe#122 protein via the Western-blot procedure using anti-pKe#122-1. To this end, cell lysates of the keratinocyte line HaCaT („HaCaT“ samples) and keratinocyte sheets were electrophoretically fractionated 8 hours after disperse treatment („NHEK 8h“ samples) in an SDS polyacrylic amide gel. The proteins were blotted on a nitrocellulose membrane according to a standard procedure. To block non-specific binding sites, incubation was performed with a 5 %w/w powdered milk/TBS buffer. Incubation at 4°C for approx. 18 hours (overnight) then was performed on the (protein) strips labeled „anti-122-1“ in a 3% powdered milk/TBS buffer with the addition of anti-pKe#122-1 antibodies (1 µg/ml), and on the (protein)strips labeled „rbIgG“ in a 3% powdered milk/TBS buffer with the addition of rabbit normal IgG (1 µg/ml). The nitrocellulose membrane was then washed with TBS/tween and TBS buffer, and incubated with an enzyme-marked anti-rabbit IgG antibody in a 3% powdered milk/TBS buffer. After renewed washing with TBS/tween and TBS, the bound antibodies were made visible with a peroxidase-specific luminescence substrate (e.g., the ECL system of Amersham-Buchler) and autoradiographically depicted. An alternative marking with chromogenic substrates is also easily possible.

[0048] The celllysates can also be directly blotted onto a nitrocellulose membrane without preceding electrophoretic fractionation and further treated as described above.

### 3. Enzyme-linked-immunosorbent-assay (ELISA)

[0049] Microtiter plates are coated with recombinant pKe#122/GST fusion protein in various concentrations (10-0 ng/ml). Non-specific binding sites are blocked via treatment with 0.1 %w/w gelatine in PBS (PBS/gelatine). The coated wells were then incubated with anti-pKe#122-1 IgG (1 µg/ml) for 1 hour at room temperature (see Fig. 6 A, closed circles). The control batch takes place with rabbit normal IgG in the same concentrations (see Fig. 6 A, open circles). After a washing step with 0.05 %v/v Tween-20 in PBS (PBS/Tween) is followed by incubation with peroxidase-marked goat-anti-rabbit IgG (1:10,000 in PBS/tween). After another washing step to remove unbound enzyme-marked antibodies, the colorless peroxidase substrate orthophenylene diamine is added, which is converted into a colored product by the peroxidase. Other peroxidase substrates with sharp color change can be used in place of orthophenylene diamine. The color formation, and hence the bound antibody, is quantified by means of an absorption measurement in a microtiter plate photometer at 490 against 405 nm (ordinate). Fig. 6A shows the result of such a test. It shows that the color concentration is proportional to the amount of pKe#122 fusion protein bound to the plate. As a result, an unknown amount of antigen can be quantified by comparison when using samples with known antigen concentrations, so-called standards.

[0050] To quantify the pKe#122 protein in complex solutions, the execution of a sandwich ELISA (Fig. 6B) is preferred. To this end, a microtiter plate is coated with an antibody oriented against pKe#122 (e.g., rabbit anti-pKe#122/GST fusion protein, 1 µg/well). The still remaining non-specific binding sites of the microtiter plate are then blocked with PBS/gelatins. The microtiter plate is subsequently mixed in with various concentrations of the pKe#122/GST protein (10-0 ng/ml). After a washing step with PBS/tween, the plate is incubated with a second peroxidase-marked anti-pKe#122 antibody (e.g., peroxidase-marked rabbit anti-pKe#122-1 (peptide) antibody) (e.g., for one hour while shaking at room temperature). „Peroxidase“ here stands for practically any marking of the antibody, e.g., with enzymes, fluorescence molecules or luminescence molecules. After an additional washing step to remove unbound, enzyme-marked antibodies, the colorless peroxidase substrate orthophenylene diamine is added, which is converted into a colored product by the peroxidase activity. The color

formation is quantified by means of an absorption measurement in a microtiter plate photometer at 490 against 405 nm (ordinate). Fig. 6B shows the result of such a test. It shows that the color concentration is proportional to the amount of pKe#122 bound to the plate. As a result, the unknown quantity of pKe#122 in a sample can be quantified by means of this test procedure. In this case, the substance orthophenylene diamine stands for any desired peroxidase substrate that detectably changes its color due to the peroxidase activity.

[0051] Instead of the polyclonal antibody „anti-pKe#122-1“ used here as an example, use can just as well be made of monoclonal antibodies, which are targeted against the protein pKe#122, namely both in the simple ELISA (=enzyme linked immunosorbent assay) and in the sandwich ELISA.

[0052] Example 4: Detection of pKe#122-specific mRNA in cells via reverse polymerase chain reaction

[0053] The polymerase chain reaction (PCR) was used to detect pKe#122-specific RNA in cells of keratinocyte sheets (NHEK) after disperse treatment and in HaCaT cells. To this end, RNA was isolated from cells of keratinocyte sheets (NHEK) after disperse treatment and incubation times of varying length, and from HaCaT cells using standard methods (guanidinium-thiocyanate-phenol-chloroform extraction method, see also Example 1A) and rewritten to cDNA according to standard methods. This cDNA was subjected to a PCR, during which a partial fragment of  $\approx 350$  kb was amplified from the pKe#122-specific cDNA. A combination of the primers „pKe#122-forward 3“ (tgagcaggcgctgggtatcatgcag) and „pKe#122-reverse 2“ (tcaccgggaacaagaaggccacct) was used as the primer pair. 10 ng of cDNA were mixed with 10  $\mu$ M of primer along with a mixture of heat-stable DNA polymerase, ATP, TTP, GTP, CTP and polymerase buffer (e.g., compare: *Current protocols in Molecular Biology*, Vol. 1, 1997, John Wiley & Sons. Inc, Suppl. 37, Chapter 15), in this example in the form of the commercially available, ready-to-use „PCR master mix“ from Clontech. In addition, the following control tests were performed: 1. The batch described above with the plasmid pUEX-1/pKe#122 instead of the cDNA; 2. The kit-internal positive control; 3. The reaction batch described above without added cDNA (negative control 1); 4. The batch described above with cDNA from cells of keratinocyte sheets (NHEK) 2 hours after disperse treatment, without adding primers (negative control 2). The reaction products of the PCR reaction were electrophoretically fractionated in agarose gel. Fig. 7 shows the results of this fractionation. The following



applies: Trace 1 = molecular weight marker; Trace 2 = NHEK T0; Trace 3 = NHEK T2; Trace 4 = NHEK T4; Trace 5 = NHEK T8; Trace 6 = HaCaT; Trace 7 = free; Trace 8 = positive control (pUEX-1; with pKe#122 as the insert); Trace 9 = negative control (batch with cDNA without primer); Trace 10 = kit-specific positive control for functional check of PCR; Trace 11 = negative control (batch with primer without cDNA); Trace 12 = molecular weight marker. A PCR product of the expected size of  $\approx$  350 kb was detected in traces 3, 4, 5, 6 and 8, meaning that pKe#122-specific mRNA was detected in cells of keratinocyte sheets at time 2 (T2), 4 (T4) and 8 (T8) hours after dispase-induced detachment, and also in HaCaT cells.

[0054] This technique makes it possible to detect the pKe#122 expression even in cases where the pKe#122 protein cannot be detected owing to excessively low expression levels by means of immune histological methods, the ELISA, dotblot or Western-blot procedures.

[0055] Example 5: Manufacture of vector molecules with the ability to express the protein pKe#122 in prokaryotic or eukaryotic cells

[0056] Two approaches were taken to manufacture or express the recombinant pKe#122 protein. In the first, two pKe#122 glutathion-S-transferase (GST) fusion proteins, pKe#122/GST-I and pKe#122/GST-II (vector pGEX; see Fig. 8) were manufactured in bacteria (*E. coli* DH5 $\alpha$ ) for purposes of expression. In the second, a pKe#122 FLAG fusion protein (vector pBK-CMV; see Fig. 9) was manufactured in eukaryotic cells (Cells-cells) for purposes of expression.

[0057] The pKe#122-gluthathion-S-transferase (GST) fusion proteins were expressed in *E. coli* (DH5 $\alpha$ ) through IPTG induction. After induction, the bacterial lysate was analyzed in a Western blot with anti-GST antibodies, specifically in comparison to pKe#122-gluthathion-S-transferase (GST) vector-carrying, non-induced bacteria lysate, and to lysate of bacteria that expressed only GST. The product of this Western-blot is shown on Fig. 10A: Trace (a) shows the control transfectant (GST without insert) before IPTG induction, trace (b) shows the control transfectant (GST without insert) after IPTG induction, trace (c) shows pKe#122/GST-I before IPTG induction, trace (d) shows pKe#122/GST-I after IPTG induction, trace (e) shows pKe#122/GST-II before IPTG induction, and trace (f) shows pKe#122/GST-II after IPTG induction.

[0058] As evident from the figure, a mixture of GST-positive bands varying in size could be detected, namely exclusively after IPTG induction. The highest molecular band had a molecular weight of approx. 100 kD (fraction 85), the lowest molecular band of approx. 26 kD, which corresponds to the molecular weight of the pure GST protein. The cited data indicate that the recombinant pKe#122-GST fusion protein breaks down in *E. coli*. The expressed fusion proteins (trace d) were present as insoluble protein aggregates in the so-called „inclusion bodies“, so that a prep-cell purification was performed. The fractions of this prep-cell purification were then electrophoretically fractionated using standard procedures, and analyzed by means of the immunoblot procedure already described above using anti-GST antibodies. The product of this purification procedure is shown on Fig. 10B. Purification resulted in a mixture of varying large GST fusion proteins. The thickest band, i.e., the majority of GST fusion proteins, had an apparent molecular weight marker of 65 kD. This allows us to conclude that the 65 kD pKe#122 GST fusion protein consists of the GST protein and an approx. 40 kD large fragment of the protein pKe#122. The 65 kD pKe#122/GST fusion protein was drawn upon to manufacture a polyclonal antiserum in rabbits. The manufacture and characterization of the antibodies took place as described in Example 2 for the anti-peptide antibodies.

[0059] The highest molecular band had an apparent molecular weight of approx. 100 kD (compare Fig. 10 B, fraction 85). This allows us to conclude that the 100 kD pKe#122-GST fusion protein consists of the GST protein and an approx. 70-75 kD large fragment of the pKe#122 protein (compare also Fig. 4 and accompanying description in Example 2).

[0060] In the eukaryotic system, the pBK-CMV-pKe#122 vector (Fig. 9) was transformed into so-called Cos cells, i.e., into cells of the Cos-cell line generally known in prior art. The Cos cells were made to absorb the plasmid-DNA in a standard procedure through treatment with DEAE-dextran/chloroquine. The transformed cells were then incubated for two days under standard conditions (37°C and 7% CO<sub>2</sub>). The Cos cells were subjected to lysis and analyzed in the immunoblot procedure using an antibody against the FLAG epitope or the anti-peptide antibody anti-pKe#122-1 IgG. Fig. 11 shows the product of the immunoblot: Trace a shows the non-transfected Cos cells, Trace b shows a FLAG control protein, and Trace c shows Cos cells transfected with pKe#122-FLAG vector construct. Trace c exhibits a band with an approximate molecular weight of 80 kD, which was stained by the anti-FLAG antibody. Trace b

shows a FLAG-marked control protein that demonstrates the functionality of the anti-FLAG antibody.

[0061] Example 6: Influencing of keratinocytes with pKe#122-specific antisense oligonucleotides

[0062] Antisense nucleotides are absorbed by cells, also keratinocytes (compare G. Hartmann et al. 1998: *Antisense-Oligonukleotide*, Deutsches Ärzteblatt 95, Issue 24, C1115-C1119), and bind to the mRNA present in the cell, inhibiting its translation, and hence expression (compare Y.-S. Lee, et al. 1997: *Definition by specific antisense oligonucleotides of a role for protein kinase C $\alpha$  in expression of differentiation markers in normal and neoplastic mouse epidermal keratinocytes*, Molecular Carcinogenesis 18, pp. 44-53). Suitable antisense oligonucleotides were manufactured using the pKe#122-specific nucleotide sequence (SEQ ID NO:1 or SEQ ID NO:4). They were set to a concentration of 100  $\mu$ M with a suitable buffer medium (so-called „oligobuffer“). HaCaT cells were cultivated at 37°C and 7% CO<sub>2</sub> up to a confluence of 70-80%. The cells were trypsinated off (10 minutes, 0.2 % EDTA, 5-10 minutes, 0.1 % trypsin) and set to a concentration of 25,000 cells/ml. 100  $\mu$ l cell suspension (corresponds to 2,500 cells) was pipetted in per well of a 96-well plate. The cells were incubated for 1 hour, followed by the addition of the antisense oligonucleotide (2  $\mu$ l of a 100  $\mu$ M solution) and further incubation for 24-48 hours. The negative control consisted of cell batches to which was added an oligonucleotide with the same base distribution, but a randomly selected sequence.

[0063] The cells treated in this manner were analyzed under a microscope for phenotypic changes in the cells. The result of the microscopic analysis is shown on Fig. 12 and Fig. 13: Fig 12 a shows sub-confluent HaCaT cultures that were treated with pKe#122-specific antisense oligonucleotides, Fig. 12 b shows sub-confluent HaCaT cultures treated with control oligonucleotides, Fig. 13 a shows confluent HaCaT cultures treated with pKe#122-specific antisense oligonucleotides, Fig. 13 b shows confluent HaCaT cultures treated with control oligonucleotides, and Fig. 13 c shows a detail section from Fig. 13 a.

[0064] The results of microscopic analysis demonstrate that, in comparison to control oligonucleotides, the number of cells in the cultures treated with the specific antisense-oligonucleotide is distinctly reduced. This allows us to conclude that the cellular proliferation was diminished by the antisense-oligonucleotide. After confluence

had been reached, the HaCaT cultures treated with antisense-oligonucleotides exhibited greatly enlarged cells, which were not discovered in the cultures treated with control oligonucleotides. These large cells correspond to differentiated keratinocytes in terms of their morphology. The findings allow us to conclude that cells treated with pKe#122-specific antisense-oligonucleotides show an increased tendency toward differentiation.

[0065] In sum, treatment with pKe#122-specific oligonucleotides has a distinct influence on proliferation and differentiation.

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